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Calsequestrin, a calcium sequestering protein localized at the sarcoplasmic reticulum, is not essential for body-wall muscle function in *Caenorhabditis elegans*

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SUMMARY

Calsequestrin is the major calcium-binding protein of cardiac and skeletal muscles whose function is to sequester Ca^{2+} in the lumen of the sarcoplasmic reticulum (SR). Here we describe the identification and functional characterization of a *C. elegans* calsequestrin gene (*csq-1*). CSQ-1 shows moderate similarity (50% similarity, 30% identity) to rabbit skeletal calsequestrin. Unlike mammals, which have two different genes encoding cardiac and fast-twitch skeletal muscle isoforms, *csq-1* is the only calsequestrin gene in the *C. elegans* genome. We show that *csq-1* is highly expressed in the body-wall muscles, beginning in mid-embryogenesis and maintained through the adult stage. In body-wall muscle cells, CSQ-1 is localized to sarcoplasmic membranes surrounding sarcomeric structures, in the regions where ryanodine receptors (UNC-68) are located. Mutation in UNC-68 affects CSQ-1 localization, suggesting that the two possibly interact in vivo. Genetic analyses of chromosomal deficiency mutants deleting *csq-1* show that CSQ-1 is not essential for initiation of embryonic muscle formation and contraction. Furthermore, double-stranded RNA injection resulted in animals completely lacking CSQ-1 in body-wall muscles with no observable defects in locomotion. These findings suggest that although CSQ-1 is one of the major calcium-binding proteins in the body-wall muscles of *C. elegans*, it is not essential for body-wall muscle formation and contraction.

Key words: *C. elegans*, Calsequestrin, Ca^{2+}-binding protein, Ryanodine receptor, RNA interference (RNAi)

INTRODUCTION

Calsequestrin is a Ca^{2+}-binding protein, first identified from the sarcoplasmic reticulum (SR) of vertebrate skeletal muscle (MacLennan and Wong, 1971). Biochemical studies revealed that calsequestrin has a moderate-affinity and high-capacity Ca^{2+}-binding activity (40-50 moles of Ca^{2+} per mole of protein, with a dissociation constant of 1 mM; MacLennan et al., 1983; Mitchell et al., 1988). Because of its high capacity and moderate affinity for Ca^{2+}, it was suggested that calsequestrin stores and concentrates Ca^{2+} in the lumen of the SR (Heilmann and Spamer, 1996; Ikemoto et al., 1989; Krause, 1991). However, the exact biological function of calsequestrin remains obscure. Calsequestrin has been found to exist in two different isoforms: a fast-twitch skeletal muscle type (Fliegel et al., 1987; Zarain-Herzberg et al., 1988) and a cardiac muscle type (Scott et al., 1988; Arai et al., 1991). Two different genes that encode each isoform have been cloned in vertebrates (Fujii et al., 1990; Park et al., 1998; Treves et al., 1992). Subsequently, calsequestrin has been identified in *Xenopus laevis* (Parys et al., 1994), in sea urchin eggs (Oberdorf et al., 1988), in *Paramecium* (Plattner et al., 1997) and in the plant *Pistia stratiotes* (Franceschi et al., 1993). So far no calsequestrin has been reported in the muscles of invertebrates. Therefore, we sought to investigate whether calsequestrin exists in muscles of the nematode *C. elegans* and to determine its function in vivo.

C. elegans is a free-living soil nematode with a relatively short life cycle. Because of its short generation time and simple body structure, *C. elegans* has been the subject of extensive genetic and cell biological studies as a useful model organism. In *C. elegans*, there are two prominent muscle types: body-wall muscle, which is used for locomotion, and pharyngeal muscle, which is used for pumping and grinding food. Many genetic and biochemical studies have suggested that body-wall and pharyngeal muscles are reminiscent of skeletal and cardiac muscles of vertebrates, respectively (Waterston, 1988).

The completion of genome sequencing combined with

forward and reverse genetic techniques has made *C. elegans* an ideal model system for investigating the gene function of vertebrate homologues. In fact, over 19,000 proteins were predicted from the nearly complete sequence and approximately 40% of these are related to proteins determined in other organisms (The *C. elegans* Sequencing Consortium, 1998). We were interested in identifying homologues of Ca^{2+}-binding proteins in *C. elegans*, especially those that may play a role in muscle contraction. Here, we report the identification and functional characterization of a gene, *csq-1*, which encodes a calsequestrin homologue in *C. elegans*. In this report we demonstrate that, although calsequestrin (CSQ-1) is predominantly expressed in body-wall muscles, it may not be absolutely necessary for body-wall muscle function in *C. elegans*. Moreover, we find that the ryanodine receptor (UNC-68) is likely to affect the proper localization of CSQ-1 in vivo.

MATERIALS AND METHODS

C. elegans strains and a cosmid clone

C. elegans strains including wild-type N2 were obtained from the *Caenorhabditis* Genetics Center (CGC) at the University of Minnesota, USA. A cosmid clone, F40E10, was obtained from A. Coulson (The Sanger Center, UK). Breeding of *C. elegans* was carried out according to Brenner (Brenner, 1974).

Cloning of calsequestrin cDNA and northern analysis

To obtain cDNA clones, two primers were designed based on the genomic sequence of F40E10.3: upstream primer (5′-ATGCGCATG-CATGACTTGGATCAAACTTGC-3′) and downstream primer (5′-ATGCAAGCTTTTACAGCTCCTTTCTAGAC-3′). Using these primers, a 1.2 kb DNA fragment was amplified from a mixed-stage worm cDNA library (kindly provided by P. Okkema and A. Fire) and cloned into pCR^{TM}II vector (Invitrogen). For northern blot analysis, total RNA was prepared as described (Krause, 1995) and polyadenylated RNAs were isolated using the micro-fast track kit (Invitrogen).

Antiserum preparation and western analysis

Complementary DNA fragments encoding the C-terminal half of the protein were subcloned into pGEX-4T1 (Pharmacia Biotech), overexpressed as GST fusion proteins in *E. coli*, purified on Glutathione-Sepharose (Pharmacia Biotech), and used to raise polyclonal antibodies in rabbit. Protein samples were prepared from staged animals by sonicating worm pellets, and resolved by SDS-PAGE. After electrophoresis, gels were stained with Coomassie Blue or transferred to nitrocellulose for western analysis. Signals were detected with HRP-conjugated goat anti-rabbit IgG (Promega).

Ca^{2+}-binding assay and 'stains-all' staining

The Ca^{2+}-binding assay was performed as described (Maruyama et al., 1984). Briefly, four different cDNA fragments that encode different regions of CSQ-1 were used to construct fusion proteins with GST. Fusion proteins overexpressed in *E. coli* were resolved by SDS-PAGE, transferred to nitrocellulose, and probed with $^{45}Ca^{2+}$. The autoradiogram of the labeled proteins was obtained by exposing the dried membrane to the imaging plate of an image analyzer (BAS-1500, Fujifilm). Ca^{2+}-binding protein was visualized by the 'Stains-all' method (Campbell et al., 1983).

Immunofluorescence microscopy and whole-mount in situ mRNA hybridization

C. elegans was immunostained as described (Ahnn and Fire, 1994; Miller and Shakes, 1995). Briefly, worms were transferred to a poly-L lysine subbed slide, permeabilized by freeze-cracking and fixed in methanol at –20°C. Fixed animals were incubated with anti-CSQ-1 diluted 1:100 in TBS-T (150 mM NaCl, 50 mM Tris-Cl, pH 7.8, 0.1% Tween 20) at room temperature for 6 hours. Samples were washed thrice for 2 minutes each in TBS-T, incubated for 6 hours with goat anti-rabbit secondary antibody (rhodamine-conjugated) and then washed as above. Slides were mounted in 80% glycerol with 1% *n*-propyl gallate to minimize bleaching. Stained specimens were observed under a fluorescence microscope (Olympus BX50). For double antibody experiments, mouse monoclonal antibody specific for vinculin (MH24) (kindly provided by Ross Francis and Michelle Hresko) and goat anti-mouse secondary antibody (FITC-conjugated) were used.

Whole-mount in situ experiments were carried out as described (Seydoux and Fire, 1994; Seydoux and Fire, 1995). Digoxigenin-labeled DNA probes were made by PCR amplification, and alkaline phosphatase-labeled anti-digoxigenin Fab fragment (Boehringer-Mannheim) was used to detect signals. Samples were prepared by the same methods used for immunostaining as described above except for the fixation step, where a fixing solution obtained from the Streck Lab. Inc. was used in place of cold methanol. Samples were viewed under a light microscope for color reactions and under a fluorescence microscope for DAPI (diaminophenolindole) staining.

Immunogold staining

Adult worms were fixed in a solution containing 2% paraformaldehyde and 0.4% glutaraldehyde (pH 7.4) for 2 hours at room temperature and washed thrice with 0.1 M PBS. After alcohol dehydration, worms were embedded in LR gold resin (Electron Microscopy Sciences) at –20°C for 72 hours under UV light. Immunogold staining was carried out as described (Yu and Chai, 1995). Briefly, sectioned specimens were washed in PBS-Milk-Tween (3% skimmed milk and 0.01% Tween 20 in PBS) and incubated for 2 hours at room temperature with primary antibody (rabbit polyclonal anti-CSQ-1). The specimens were then washed thoroughly in PBS-BT (1% bovine serum albumin and 0.01% Tween 20 in PBS) and reincubated overnight at 4°C with 5 nm gold-conjugated goat anti-rabbit IgG (BioCell). Samples were washed in PBS-Tween and stabilized with glutaraldehyde for 10 minutes. For silver enhancement, a commercial kit was used (Amersham) and the background was stained with uranyl acetate and lead citrate. Samples were air-dried and examined under a transmission electron microscope (Jeol 1200 EXII).

DNA and RNA microinjection

The 5′ upstream region of the *csq-1* gene (2.4 kb) was cloned by PCR and fused to *gfp* (green fluorescent protein) reporter genes (Fire et al., 1998a) to construct expression plasmids (*C. elegans* expression vector kit was provided by A. Fire). Plasmid DNA was microinjected into wild-type animals to obtain germline transformants (Mello and Fire, 1995). As a transformation marker, plasmid pRF4 containing a dominant gene (*rol-6*) was coinjected.

For RNA interference (RNAi) experiments, a 348-bp *Eco*RV cDNA fragment (amino acid residues 33-148) and a 522-bp *Eco*RI-*Xba*I cDNA fragment (amino acid residues 267-440) of *csq-1* gene were used to prepare double-stranded RNAs (dsRNAs). dsRNAs were also prepared to target GFP and RYR (UNC-68). For *unc-68*, two *Eco*RV cDNA fragments, one 574 bp (amino acid residues 1-191) and the other 1278 bp (amino acid residues 1190-1615) were used. For *gfp*, pPD79.44 GFP vector was used. Double-stranded RNAs were prepared using an in vitro transcription kit (Promega) as described (Fire et al., 1998b; Montgomery et al., 1998; Timmons and Fire, 1998). RNA injections (1 μg/μl) were carried out in the gonads of the adult hermaphrodites as described (Mello and Fire, 1995). After recovery in bacteria-seeded worm agar plates, the injected worms were transferred to fresh worm plates at 16-hour intervals. The

progeny of injected animals were observed for phenotype and were used for staining or protein preparation.

Analysis of phenotypes in animals subjected to RNAi

Interference with *csq-1* and *unc-68* was assayed in a wild-type (N2) and an *unc-68 (e540)* mutant background. Phenotypes analyzed included viability, brood size, growth rates, body shape and moving speeds. We videotaped CSQ-1 deficient animals and control animals under a low magnification stereomicroscope. Moving speed was measured by reviewing the videotape and calculating the average distance traveled by an individual. Levamisole sensitivity was assayed by examining the time for complete paralysis of the worms when exposed to 100 μM levamisole solution in microtiter wells. Interference with *gfp*, a gene whose product is not found endogenously in *C. elegans*, was assessed using the GFP expression vector, pPD79.44. The phenotypes of the F_1 progeny were examined under a dissecting microscope.

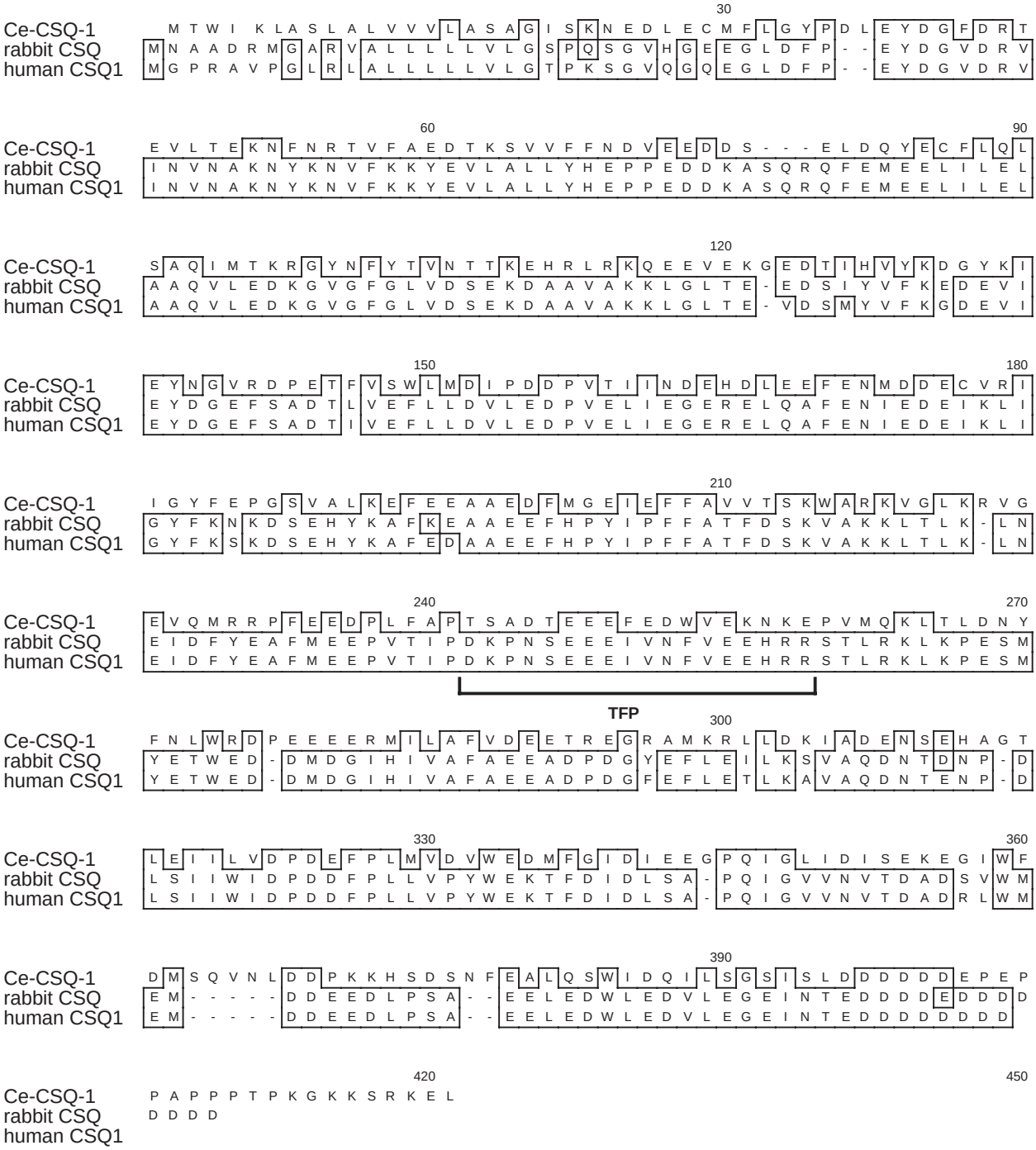

Fig. 1. Amino acid sequence alignment of *C. elegans* calsequestrin and vertebrate calsequestrins. The predicted protein sequence of *C. elegans* CSQ-1 (Ce-CSQ-1) is depicted and aligned with rabbit skeletal (rabbit CSQ, Accession No. M15747) and human skeletal (human CSQ-1, Accession No. AA197255) calsequestrins. Regions of identity among calsequestrin homologs are boxed. The putative trifluoperazin (TFP) binding site is shown by the bold line.

RESULTS

Identification of single calsequestrin gene (*csq-1*) in *C. elegans*

To investigate whether calsequestrin, one of the major calcium-binding proteins in muscle cell, exists in *C. elegans*, we searched the worm genome database for calsequestrin-like sequences. In contrast to vertebrates, which have two isoforms of calsequestrin encoded by two different genes, only one calsequestrin gene (*csq-1*) sequence was found in *C. elegans*, and this contained an open reading frame of 417 amino acids. The deduced amino acid sequence showed approximately 50% similarity (30% identity) to that of rabbit skeletal calsequestrin (Fig. 1). Although the sequence similarity was moderate, the similarity existed throughout the entire length of the protein including many conserved amino acids. In addition, *C. elegans* calsequestrin (CSQ-1) showed several other characteristics of calsequestrins. First, it appeared to have a signal peptide sequence at the amino terminus. Second, in the carboxy-terminal region, it contained a stretch of aspartic acid residues. Third, it also contained a motif, K-XX-S/T-EEE-L/I-XX(X)-F-XXXX-R, which is involved in specific protein-protein interactions and is known to be a high-affinity binding site for the drug, trifluoperazine (Yano and Zarain-Herzberg, 1994), which decreases the maximum Ca^{2+} binding by calsequestrin (He et al., 1993).

The *csq-1* gene is located on cosmid F40E10 that has been physically mapped to the right arm of the X chromosome (Fig. 2A) and corresponds to the region between the *his-24* and the *unc-3* loci on the genetic map (Herman, 1987; Sanicola et al., 1990). Analysis of the genomic sequence indicated that the coding region of *csq-1* consists of 10 exons (Fig. 2B). There are two available chromosomal deficiencies, *mnDf4* and *mnDf10* (Meneely and Herman, 1979), deleting regions containing the *csq-1* gene (Fig. 2A). In the smaller *mnDf10* mutant a chromosome region of approximately 3 cM covering *csq-1* is deleted. We have previously characterized embryos homozygous for the *mnDf10* deficiency, which were arrested at the twofold stage and showed body-wall muscle filament formation (Ahnn and Fire, 1994; Lee et al., 1997a). Additionally, these embryos showed muscle twitching similar to wild-type and pharyngeal structure formation (Lee et al., 1997a). These data suggest that embryos that lack the *csq-1* gene are still capable of initiating functional body-wall muscle formation and contraction. Since the deficiency mutation deletes many genes other than *csq-1*, it was not possible to assess the phenotypes for *csq-1* alone in the region deleted by the deficiency. However, if *csq-1* is required zygotically for the initiation of body-wall muscle formation, the embryos of a homozygous deficiency should have failed to initiate body-wall muscle formation. Thus, it is likely that zygotic *csq-1* is not essential for the initiation of body-wall muscle formation and contraction.

The *csq-1* gene encodes a calcium-binding protein

Vertebrate calsequestrin is known to have moderate affinity and high capacity for Ca^{2+} binding (Mitchell et al., 1988). Because CSQ-1 showed sequence similarities with other calsequestrins, we sought to determine whether CSQ-1 could bind Ca^{2+}. Four regions of CSQ-1 (N-terminal half, N-terminal three-quarters, C-terminal half and full length) were overexpressed as fusion proteins with GST (glutathione-S-transferase) in *E. coli* (Fig. 3A,B). Calcium overlay experiments using $^{45}Ca^{2+}$ showed that all of the recombinant calsequestrin proteins had calcium-binding activities (Fig. 3C). The GST protein itself did not bind calcium, whereas an SR preparation from rat heart that contains a cardiac calsequestrin as the major protein showed a calsequestrin-specific signal (Fig. 3C). To further visualize calcium-binding proteins and to confirm that CSQ-1 binds Ca^{2+}, the 'stains-all' method was used (Campbell et al., 1983). The full-length CSQ-1 was stained blue on an SDS-PAGE gel (data not shown). Taken together with the calcium overlay experiment, we conclude that *csq-1* encodes a protein that possesses calcium binding activities.

Zygotic transcripts of the *csq-1* appear in the body-wall muscle cells throughout the development of *C. elegans*

To determine the temporal and spatial expression of *csq-1*, we performed northern blot analysis and whole-mount in situ hybridization experiments (see Materials and Methods). Northern blot analysis revealed a single transcript of 1.5 kb detectable at all developmental stages (Fig. 4A). During the embryonic stages a very weak signal was detected, which

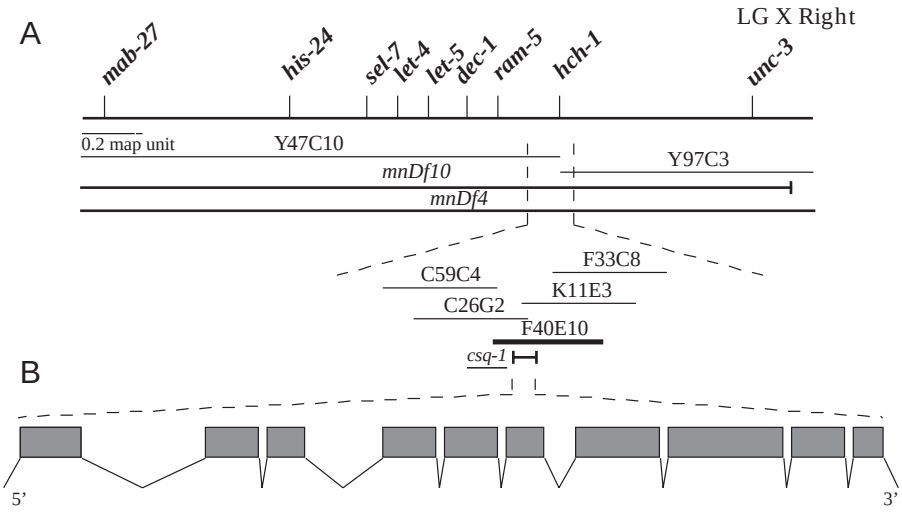

Fig. 2. Molecular nature of *csq-1*. (A) Genetic and physical maps of *csq-1* region. The position of *csq-1* relative to the nearby genetic markers, deficiencies, and regions covered by the yeast artificial chromosomes, on the right end region of LG X is shown. *Csq-1*, which is mapped to the cosmid F40E10 (Accession No. Z69792) and the neighboring cosmids are indicated. (B) The predicted F40E10.3 gene is composed of 10 exons (shaded boxes) separated by introns.

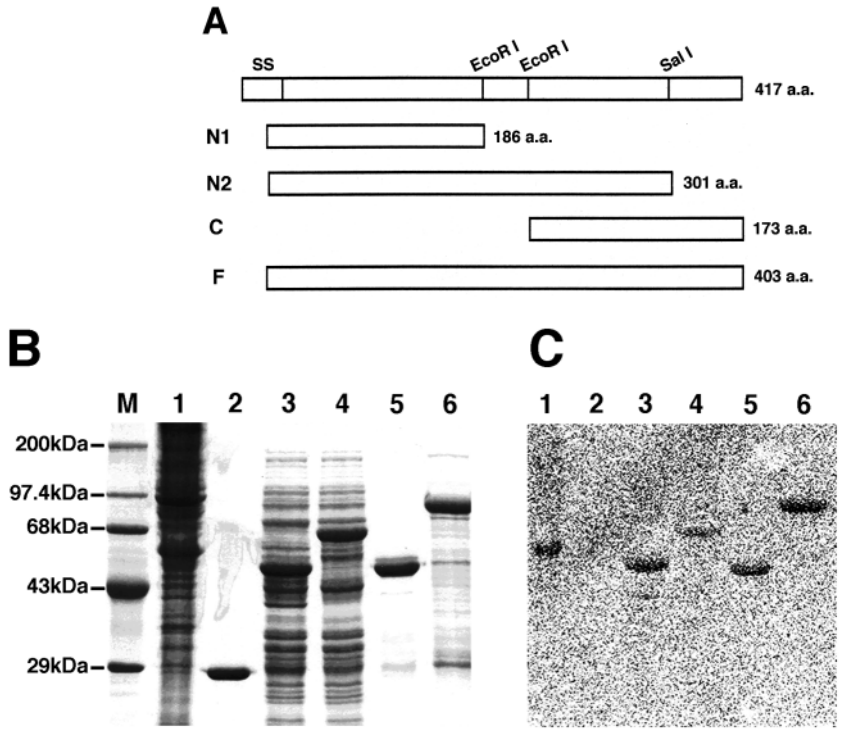

Fig. 3. Calcium binding assay. (A) Four cDNA fragments that encode different regions of CSQ-1 were used: N-terminal half (N1, 186 aa), N-terminal three-quarters (N2, 301 aa), C-terminal half (C, 173 aa), and full-length with truncated signal peptide (F, 403 aa). Restriction sites used are shown above and SS indicates the signal peptide sequences. (B) GST-fusion proteins were overexpressed in *E. coli*, resolved by SDS-PAGE and stained with Coomassie Blue. (C) Autoradiogram of $^{45}Ca^{2+}$ overlay. Bands indicate the $^{45}Ca^{2+}$-labeled over-expressed proteins. Lane M, size marker; lane 1, SR preparation from rat heart; lane 2, GST; lane 3, N1; lane 4, N2; lane 5, C; lane 6, F.

increased significantly during larval and adult stages. In situ hybridization experiments with anti-sense probe showed that the *csq-1* gene began to be expressed in twofold-stage embryos along their mid-body lines where body-wall muscle precursor cells are located (Fig. 4B). No signal was detected in embryos earlier than the twofold stage, suggesting that the *csq-1* gene is not expressed maternally. All the embryos in stages later than twofold showed *csq-1* expression in their body-wall muscle precursor cells. Larvae and adult animals showed robust signals in body-wall muscle cells (Fig. 4C-E), whose nuclei could be identified by DAPI staining (Fig. 4F). In control hybridization experiments with a sense-strand probe no signal was detected (data not shown).

Localization of calsequestrin proteins in body-wall muscle is affected by the absence of ryanodine receptor channels

Anti-CSQ-1 antibody detected a single band migrating as a 64 kDa protein in western blots of *C. elegans* extracts (Fig. 5). The predicted molecular mass of CSQ-1 is 47 kDa. This apparent difference between the predicted molecular mass and the estimated molecular mass on SDS-PAGE is one of the characteristics of calcium binding proteins and has also been observed for other vertebrate calsequestrins (Fliegel et al., 1987; Scott et al., 1988), but the exact molecular nature of this discrepancy is still unclear. Western blot analysis showed that CSQ-1 was detectable at all stages of the development, which is consistent with the northern and in situ results (Fig. 4A-D). During larval stages, CSQ-1 levels appeared to increase compared to the embryonic stages and these higher levels were maintained through the adult stages (Fig. 5B).

To localize CSQ-1 in situ, we stained whole-mount wild-type *C. elegans* with anti-CSQ-1 antibodies. A mesh-like staining was observed in the body-wall muscle cells (Fig. 6A). The CSQ-1 staining pattern was further compared with that of UNC-68 (Maryon et al., 1998). The *unc-68* gene encodes a ryanodine receptor channel (RYR), which regulates contraction of muscle by gating the release of Ca^{2+} from SR (Coronado et al., 1994; Maryon et al., 1996; Sakube et al., 1997). Interestingly, the staining pattern of CSQ-1 looked very similar, if not identical, to that of UNC-68 antibody staining (Maryon et al., 1998 and Fig. 6B), which also showed a mesh-like pattern, in addition to punctate staining, in body-wall muscle cells. Since in vitro biochemical studies suggested that ryanodine receptor interacts with calsequestrin and other proteins in the SR (Guo and Campbell, 1995), we sought to test the possibility that CSQ-1 interacts with RYR. In order to examine this, *unc-68(e540)* mutant animals were stained with anti-CSQ-1 antibodies (Fig. 6C). Interestingly, the mesh-like CSQ-1 staining decreased greatly in these mutants and was replaced by punctate staining (compare Fig. 6C to A). The *unc-68(e540)* mutants have a mutation at the splicing acceptor site of the gene encoding the RYR, which behaves like a null mutation (Maryon et al., 1996; Sakube et al., 1997). The specificity of CSQ-1 staining in the *unc-68* mutant background was confirmed by a similar punctate staining (data not shown) in the *unc-68(r1161)* mutant allele stained with anti-CSQ-1 antibodies. This change from a mesh-like staining pattern (Fig. 6A) to a much reduced and punctate staining (Fig. 6C) of CSQ-1 seemed to be specific for *unc-68* mutants because two other uncoordinated mutants, *unc-29(e1072)* and *unc-6(e78)*, when stained with anti-CSQ-1 antibodies, revealed the mesh-like pattern similar to wild-type (Fig. 6D and data not shown). These results indicate that CSQ-1 localization is dependent on the presence of RYRs.

To further localize CSQ-1 within the muscle cells, we conducted double-antibody staining in N2 wild-type worms with anti-CSQ-1 antibodies and monoclonal antibodies specific for the myofilament lattice protein, vinculin. Vinculin is a

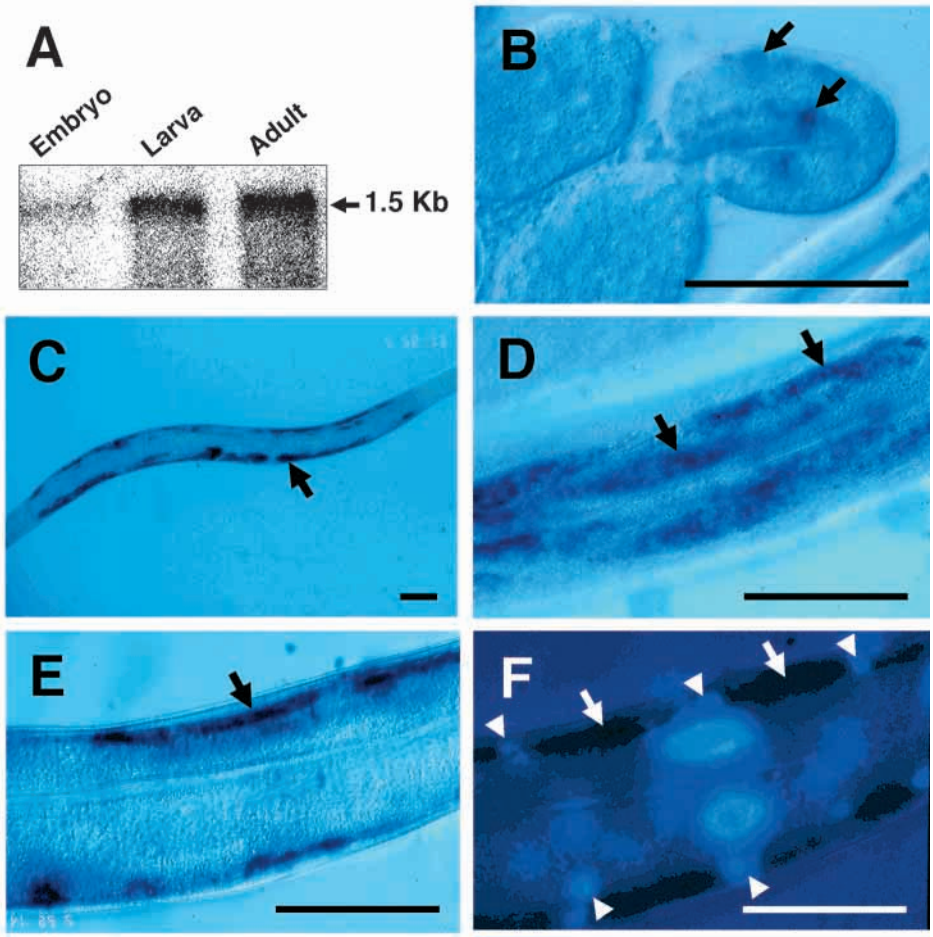

Fig. 4. Northern blot analysis and whole-mount in situ hybridization. (A) mRNA levels of *csq-1* at different developmental stages were assayed. Equal amounts (1 μg) of poly(A)$^+$ RNA were loaded in each lane and hybridized with a ^{32}P-labeled *csq-1* cDNA probe. A transcript of approx. 1.5 kb is observed in all three lanes. (B-E) In situ localization of *csq-1* RNA at different developmental stages, viewed under a light microscope (Nomarski optics). Antisense probes show *csq-1* expression (arrows) along the mid-body line in the twofold stage embryo (B), in body-wall muscle cells in the larva (C) and in the adult (D,E). (F) DAPI stained nuclei of the muscle cells showing *csq-1* expression. Arrows indicate cytoplasmic signals and arrowheads indicate nuclei of body-wall muscle cells. Bars, 50 μm.

component of dense body structures, the attachment points for thin filaments, and is found near the sarcolemma (Barstead and Waterston, 1989; Moulder et al., 1996). The mesh-like signals of CSQ-1 (red) overlapped with the punctate vinculin signals (green/yellow) (Fig. 7A-C). Maryon et al. (Maryon et al., 1998) observed punctate vinculin signals alternating with the UNC-68 signals and showed the localization of UNC-68 between the rows of dense bodies, in the thick filament or A-band region. Although the double-labeling of body-wall muscles with anti-CSQ-1 and anti-vinculin show that both the proteins are localized around the same areas (Fig. 7B), the strong mesh-like staining of CSQ-1 spanning the muscle bands indicates that CSQ-1 is expressed over a wider range in the myofilament lattice.

We also undertook a study of the subcellular localization of calsequestrin by conducting immunogold electron microscopy (EM) (see Materials and Methods). In body-wall muscle cells of wild-type animals, CSQ-1 signals were observed in the cytoplasmic regions of muscle cells, at sarcoplasmic membranes surrounding myofibril bundles, and also at spaces between sarcomeric bundles, where the dense body structures could be observed (Fig. 8A,B). Clustered signals were

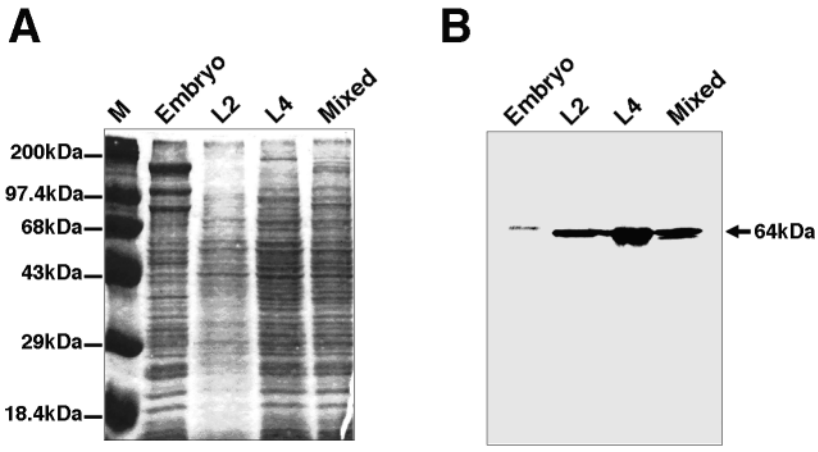

Fig. 5. Western blot analysis of calsequestrin expression during development. (A) A Coomassie Blue-stained SDS-PAGE gel showing the total protein profiles of different developmental stages: embryo, L2 larvae, L4 larvae, and mixed-stage worms. (B) An identical gel was used to transfer proteins onto a nitrocellulose membrane and immunoblotted with anti-CSQ-1. A 64 kDa band is detectable at all stages of development. A weak signal is detected in the embryo, and this increased in the larval stages.

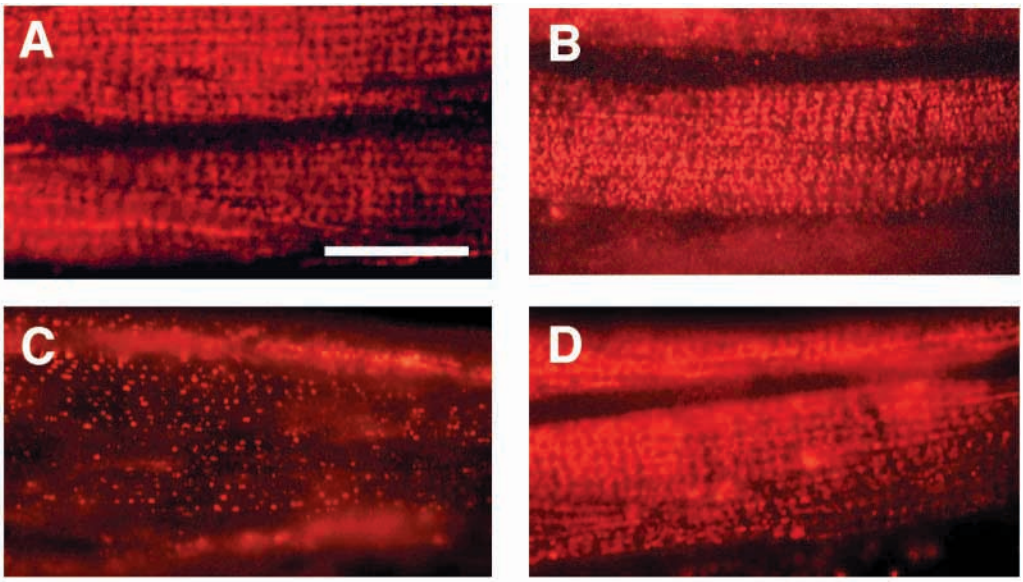

Fig. 6. Immunostaining of wild-type animals and *unc-68*(*e540*) mutants. Wild-type adult worms stained with CSQ-1 antibody (A) show mesh-like staining, and with UNC-68 (ryanodine receptor channel) antibody (B) show the punctate and mesh-like staining in body-wall muscles. (C) An *unc-68*(*e540*) mutant stained with anti-CSQ-1 antibody shows a dispersed and reduced punctate pattern. (D) An *unc-29(e1072)* mutant stained with anti-CSQ-1 antibody shows a wild-type staining pattern. Bar, 20 μm.

observed at the apical SR membranes juxtaposed to the basement membranes. Interestingly, these regions of 'flattened SR-like vesicles' have already been reported to be the sites for the localization of RYR (UNC-68) (Maryon et al., 1998), suggesting that CSQ-1 may be localized at the SR membranes in the regions where RYR are located.

In *unc-68(e540)* mutant, CSQ-1 appeared to localize mainly in the cytoplasmic regions, and the signals in the apical plasma membrane were significantly reduced but not completely absent (Fig. 8C,D). Control experiments with *unc-29(e1072)*, which has a mutation in the gene encoding a non-alpha subunit of nicotinic acetylcholine receptor (Fleming et al., 1997), showed no change of CSQ-1 signals in the apical sarcoplasmic membranes (Fig. 8E,F). This observation is in agreement with the immunostaining pattern (Fig. 6B,C) in which the mesh-like pattern of CSQ-1 is absent in *unc-68* mutants but present in *unc-29* mutants. Taken together, these results confirm that SR localization of CSQ-1 in vivo requires or is affected by UNC-68, and suggest that these two proteins may interact with each other in vivo.

Csq-1 is also expressed in vulval muscle and pharyngeal muscle cells

To visualize the expression of *csq-1* gene, we used the *gfp* reporter gene. Transgenic animals carrying the *csq-1::gfp* fusion constructs showed expressions of GFP in body-wall muscles beginning from the twofold stage embryo through to adult stages (Fig. 9A-C), which is consistent with the in situ and northern blot results (Fig. 4A-E). In addition, GFP signals were observed in vulval muscles and in the isthmus and terminal bulb regions of the pharynx (Fig. 9D-F). It is worth noting that *C. elegans* RYRs have also been shown to be expressed in vulval muscles and in the isthmus and terminal bulb of the pharynx in addition to body-wall muscles (Maryon et al., 1998; Sakube et al., 1997).

Tissue-specific interference of calsequestrin expression

In order to assess the function of the *csq-1* gene directly, we performed dsRNAi (see Materials and Methods) and examined

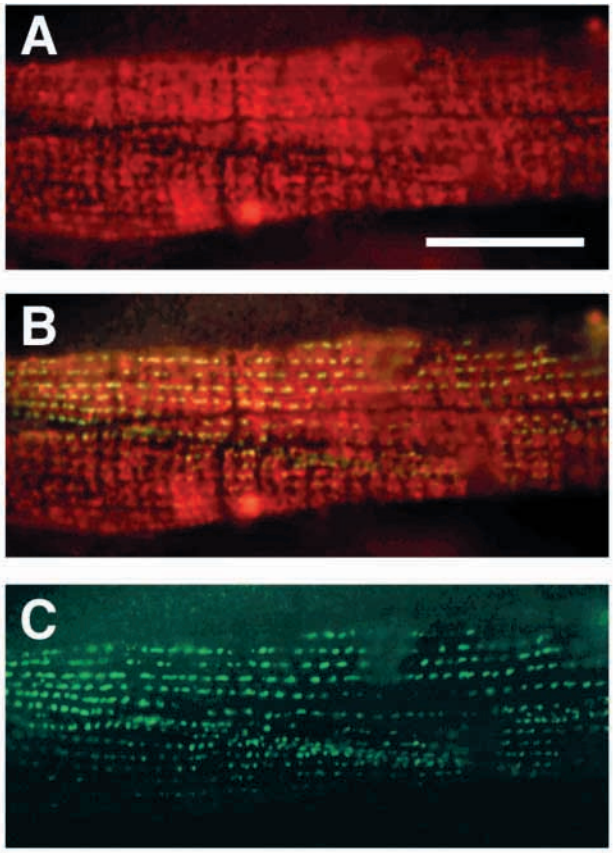

Fig. 7. Double-labeling with anti-CSQ-1 polyclonal antibodies and monoclonal antibodies specific for vinculin. Anti-CSQ-1 antibody staining is shown in red and anti-vinculin staining is shown in green/yellow. (A-C) Wild-type body-wall muscle stained with anti-CSQ-1 (A), anti-CSQ-1 and anti-vinculin (B) and anti-vinculin (C). Bar, 20 μm.

the effects of interference at the cellular level. In *C. elegans*, dsRNAs have been shown to interfere with gene expression of a target gene in a sequence-specific manner (Fire et al., 1998b;

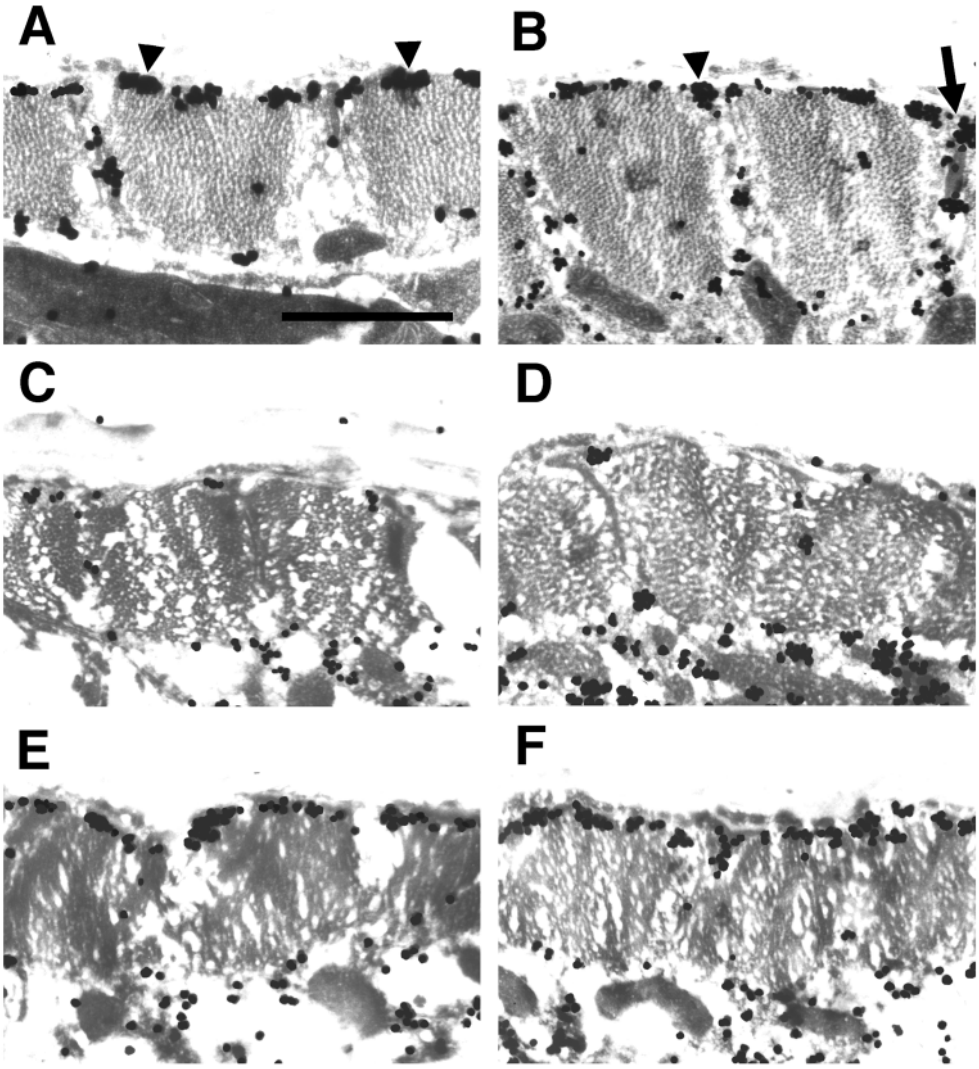

Fig. 8. Electron micrograph showing the localization of CSQ-1 in muscle cells by immunogold staining. (A,B) Transverse section of the body-wall muscle structure of wild-type worms showing CSQ-1 localization, as indicated by the gold particle signals in the cytoplasmic regions of muscle cells, apical SR membrane (arrowhead), and around the dense body region (arrow). (C,D) Localization of CSQ-1 in *unc-68(e540)* mutant, showing reduced signals in the apical SR membrane. CSQ-1 appeared to localize mainly in the cytoplasm. (E,F) Localization of CSQ-1 in *unc-29(e1072)* mutant is similar to wild-type, as seen in A and B. Bar, 1 μm.

Montgomery and Fire, 1998; Timmons and Fire, 1998). As a control, *gfp* dsRNA was injected into the *csq-1::gfp* line. Interestingly, GFP expression in body-wall muscle was completely absent, whereas expression in the vulval and pharyngeal muscles persisted (Fig. 9D-F). Tissue-specific interference had already been observed elsewhere, where non-striated vulval muscles appeared to be resistant to dsRNA interference (Fire et al., 1998b). The underlying mechanism for such tissue-specific interference is not known. All progeny of injected worms showed a complete lack of GFP expression in body-wall muscles, suggesting that the interference was highly specific and efficient in this tissue.

Next, the progeny of *csq-1* dsRNA-injected wild-type worms were characterized. First, we tested whether body-wall muscle expression of CSQ-1 had been reduced, as observed in the GFP control experiments. When progeny of dsRNA injected animals were stained with anti-CSQ-1 antibody, no CSQ-1 staining was detected in body-wall muscle; in contrast CSQ-1 expression in pharyngeal and vulval muscles appeared unchanged (Fig. 9G-I).

To examine the level of CSQ-1 protein in vivo, we performed western blot analysis with the extracts of progeny from the *csq-1* RNAi worms. Since the body-wall muscle comprises the major tissue in *C. elegans*, we expected a substantial change in the level of CSQ-1. Indeed, RNA injections significantly reduced CSQ-1 protein (Fig. 10A) but did not affect the levels of another calcium binding protein, calreticulin (CRT) (Fig. 10B). Densitometric analysis revealed that dsRNA injection reduced CSQ-1 levels by 85%, consistent with the observation that CSQ-1 expression in body-wall muscles was completely eliminated (Fig. 9G). The low level of CSQ-1 protein (Fig. 10A) was probably due to CSQ-1 expression in the pharyngeal and vulval muscles, which have shown to be resistant to dsRNA interference (Fig. 9H,I; Fire et al., 1998b). Taken together, these data indicate that, irrespective of the sequences chosen for making *csq-1* RNA, RNAi was sufficient to eliminate CSQ-1 expression in body-wall muscles although residual expression was observed in other tissues.

Characterization of progeny with calsequestrin-deficient body-wall muscles

Our results show that calsequestrin is predominantly expressed in the body-wall muscle cells of *C. elegans* and it seemed probable that it has an important role in excitation-contraction coupling (E-C coupling) during locomotion. On the other hand, genetic data on chromosomal deficiency mutants deleted for *csq-1* revealed that *csq-1* is not essential for the initiation of functional body-wall muscle formation (Ahnn and Fire, 1994; Lee et al., 1997a). Since RNAi specifically obliterated CSQ-1 in the body-wall muscles (Fig. 9G), we were interested to determine the function of CSQ-1 in this tissue. Progeny of *csq-1* (RNAi) animals, which had no detectable CSQ-1 in the body-wall muscles, were characterized and compared with wild-type worms.

As described in Table 1, normal viability and growth were

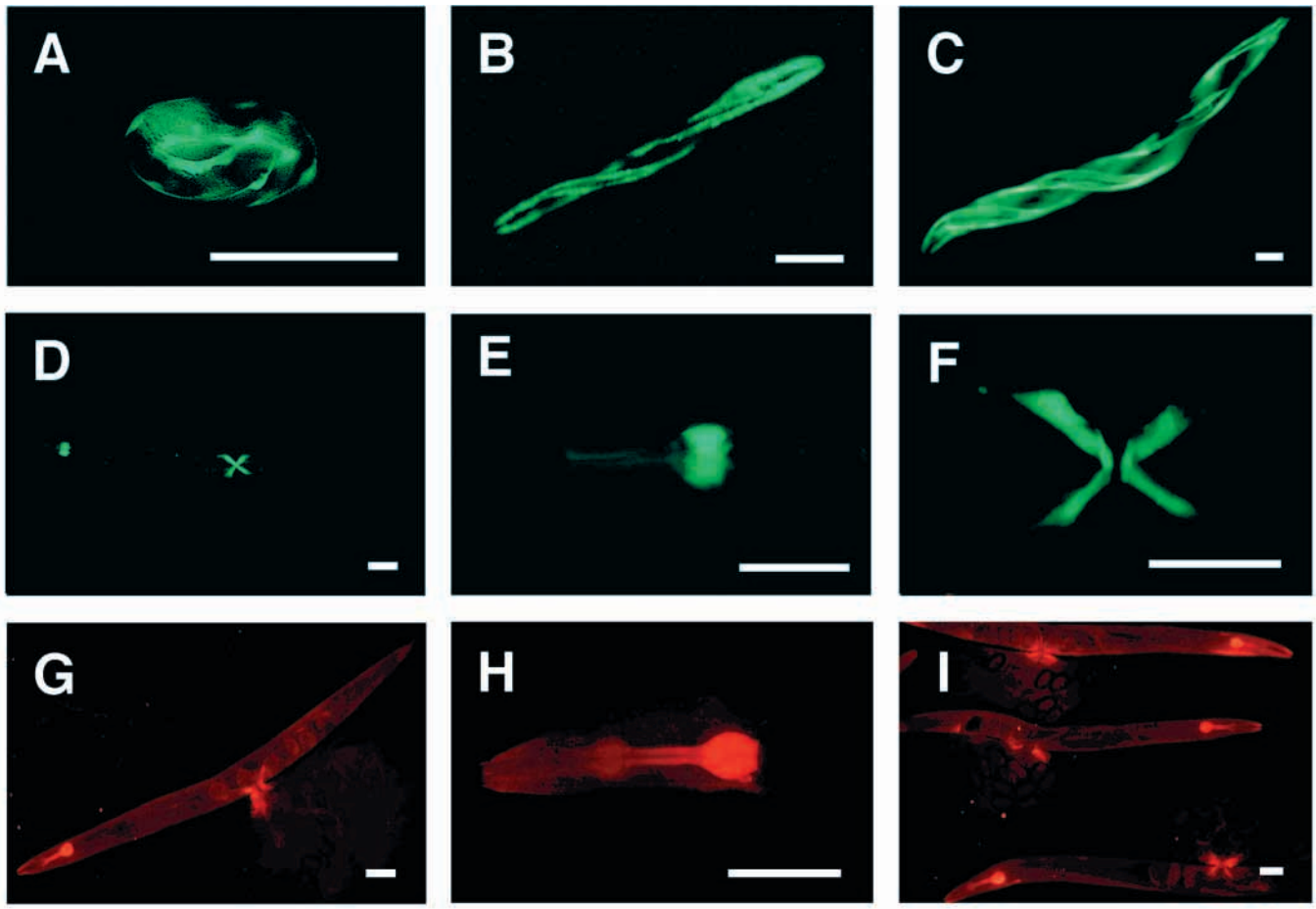

Fig. 9. GFP expression under the control of the *csq-1* promoter. Live transgenic animals were observed under a fluorescence microscope. (A) A threefold-stage embryo inside the eggshell shows GFP expression in body-wall muscle. (B) A larva showing body-wall muscle cells expressing GFP. (C) An adult showing GFP expression in body-wall muscles from anterior head (left) to posterior tail (right). The twist of the body is due to the roller phenotype of this transgenic animal. (D) An adult worm of a *csq-1*::*gfp*-expressing line after injection of *gfp* dsRNA, showing residual expression in vulval and pharyngeal muscles. Body-wall muscle expression has been severely reduced by RNAi. Magnified view of pharyngeal (E) and vulval muscle (F) expression. (G-I) Progeny of wild-type worms injected with *csq-1* dsRNA and immunostained with anti-CSQ-1 antibody, showing residual CSQ-1 expression in pharyngeal and vulval muscles only. Body-wall muscle expression has been severely reduced by RNAi, as seen in D. Bars, 50 μm.

observed in the CSQ-1 deficient animals. Additionally, these animals had normal brood size. Control experiments with *gfp* dsRNA also resulted in a wild-type phenotype. We next checked for abnormalities in locomotion in *csq-1* (RNAi) animals. To our surprise, there were no significant differences between wild-type and the CSQ-1 deficient animals as both exhibited similar moving speeds (Table 1). When dsRNAs targeted to *unc-68* were injected in a wild-type background, phenotypes associated with *unc-68* mutants themselves were observed, which indicates that the *unc-68* RNAi may phenocopy the mutant phenotype (Table 1). It also suggests that the methods used for RNAi are effective for another SR protein. We then examined the effect of levamisole, an agonist for nicotinic acetylcholine receptors that causes hypercontraction of body-wall muscle. Many uncoordinated (Unc) mutants including *unc-29* and *unc-68* are characterized by levamisole insensitivity (Lewis et al., 1980a; Lewis et al., 1980b). As shown in Table 1, wild-type animals and and CSQ-1-deficient worms become paralyzed in similar time periods, indicating that both are sensitive to the drug to the same degree. In contrast, *unc-68* mutants show a certain degree of insensitivity to levamisole, as previously reported (Lewis et al., 1980a; Lewis et al., 1980b).

To examine any effect due to loss of CSQ-1 in a sensitive Unc background, *csq-1* dsRNAs were injected into the *unc-68(e540)* animals. Resulting progeny were shown to exhibit

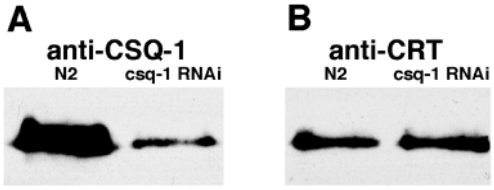

Fig. 10. Western blot analysis of CSQ-1 expression in *csq-1* (RNAi) progeny. (A) Drastic reduction in the level of CSQ-1 in RNA injected worms compared to the N2 wild-type worms. (B) An identical blot was immunoblotted with a control antibody, calreticulin (anti-CRT), where the levels of calreticulin remain unchanged in *csq-1* (RNAi) worms.

Table 1. Effects of dsRNAi on the progeny of wild-type N2 and *unc-68 (e540)* mutant hermaphrodites

Genotype of injected animal	Target protein for RNAi*	Speed (μm/second)‡	Phenotype of F_1 progeny	Average time (minutes) for complete paralysis in 100 μM levamisole‡
N2 (wild type)	None	172±18	Wild type	47±19
N2 (wild type)	Green fluorescent protein (GFP)	169±17	Wild type	51±20
N2 (wild type)	Calsequestrin	167±20	Wild type	52±18
N2 (wild type)	Ryanodine receptor	68±18	Thin body shape, weak kinker	113±30
unc-68	None	65±13	Thin body shape, weak kinker	106±25
unc-68	Calsequestrin	70±9	Thin body shape, weak kinker	102±24

*dsRNAs corresponding to *gfp*, *csq-1* and *unc-68* were injected into the wild-type and *unc-68* mutant hermaphrodites at a concentration higher than 1μg/μl for each of the genes.

‡At least 35 animals were microinjected, and the resulting progeny were observed for their phenotypes. Out of these progeny at least 40 were randomly picked to measure the speed and the time for paralysis.

mild uncoordinated movement with thin body shape and weak kinker phenotypes (Table 1), the phenotypes generally seen for *unc-68(e540)* mutants alone. These results indicate that abolition of CSQ-1 expression in body-wall muscle by RNA interference did not cause any gross phenotypic defect including locomotion, and did not show any additive or synergistic effect even in the *unc-68* mutant background. Taken together, these data suggest that CSQ-1, which is predominantly expressed in body-wall muscles, may not be required for body-wall muscle specific locomotory functions in *C. elegans*.

DISCUSSION

In this study, we have identified and characterized a *C. elegans* homologue of calsequestrin, a calcium sequestering protein localized at the SR. The *C. elegans* calsequestrin (CSQ-1) showed approximately 50% amino acid sequence similarity with rabbit skeletal calsequestrin, and showed calcium binding activity when recombinantly expressed in *E. coli* (Fig. 3C). Southern blot analysis (data not shown) and the genome sequence database further confirmed that a single gene of calsequestrin (*csq-1*) exists in *C. elegans*, in contrast to vertebrates that have a cardiac and fast-twitch skeletal calsequestrins encoded by two different genes. This is the first case of calsequestrin to be found in invertebrate muscles, although calsequestrin-like proteins have already been reported in sea urchin embryos (Oberdorf et al., 1988).

Components in E-C coupling of muscle contraction

E-C coupling converts an electrical signal of membrane depolarization to a Ca^{2+} signal that triggers muscle contraction (Bagshow, 1993). Voltage-gated Ca^{2+} channels (VGCCs) in the plasma membrane and ryanodine receptors (Ryrs) localized at the SR are known to mediate E-C coupling in striated muscles (Caterall, 1991). Calsequestrin has been suggested to be one of the downstream components in the E-C coupling machinery by two lines of thought. First, in vitro interaction between Ryrs and other proteins, including calsequestrin, was demonstrated (Zhang et al., 1997). Second, maintaining a high Ca^{2+} concentration in the SR is important for Ca^{2+} release from the SR by Ryrs. Calsequestrin has a high Ca^{2+}-binding capacity (Mitchell et al., 1988; Wang et al., 1998) and hence was suggested as the major Ca^{2+} storage reservoir inside the SR.

This E-C coupling machinery seemed to be conserved in metazoans, including *C. elegans* (Maryon et al., 1998). In *C. elegans*, the *egl-19* gene, which encodes the α1 subunit of an L-type voltage-gated Ca^{2+} channel, has been shown to be essential for E-C coupling (Lee et al., 1997b). Ryanodine receptor channel (RYR) encoded by *unc-68* (Maryon et al., 1996; Sakube et al., 1997), is another Ca^{2+} channel located at the SR. The null mutants *unc-68(r1162)* show pharyngeal abnormalities and an incomplete flaccid paralysis phenotype but the body-wall ultrastructure is normal (Maryon et al., 1996). Although *unc-68* null mutants are impaired in locomotion, coordinated contraction is propagated in body-wall muscle, suggesting that there may be UNC-68 independent contraction in *C. elegans* (Maryon et al., 1998).

Ryanodine receptor may be important for calsequestrin localization at the SR

We have shown that a mesh-like pattern of CSQ-1 staining changes to a dispersed, punctate form in *unc-68* mutants but not in other Unc mutants (Fig. 6C,D). Consistent with this observation, immunogold EM revealed that CSQ-1 failed to localize at the apical regions of the SR in *unc-68* mutants (Fig. 8C,D). Taken together, these data suggest that the absence of ryanodine receptors in *unc-68* mutants affect the localization of CSQ-1 at the apical regions of the SR. Complex formation between Ryr and other SR proteins, including calsequestrin, has been previously reported in canine cardiac muscle preparations (Zhang et al., 1997). These biochemical experiments suggest that junctin, triadin, calsequestrin and Ryr form a quaternary complex that may be required for Ca^{2+} release during E-C coupling. Our immunostaining and immunogold EM data show that UNC-68 expression is necessary for proper localization of CSQ-1 in body-wall muscle cells. Although this finding is consistent with a model in which nematode calsequestrin also associates with UNC-68 and other SR proteins, the existence of this complex in *C. elegans* has not been directly demonstrated. However, further experiments are essential to prove the interaction more directly.

Function of calsequestrin in body-wall muscle cell in *C. elegans*

Apart from storing high concentrations of Ca^{2+} in the muscle SR that is necessary for E-C coupling (Heilmann and Spamer, 1996), a regulatory function for calsequestrin has been suggested. Depending on its phosphorylation state, calsequestrin selectively controls channel activity of Ryr (Szegedi et al., 1999). In vivo function of calsequestrin has been further reported in transgenic mice where cardiac calsequestrin overexpression caused depressed cardiovascular function and hypertrophy (Sato et al., 1998).

That CSQ-1 is expressed in muscle was originally suggested by our experiments showing that an upstream region of the *csq-1* promoter drives muscle-cell specific expression of a GFP reporter gene (Cho et al., 1999). We have now generated nematodes in which calsequestrin expression is severely reduced in body-wall muscles by RNAi. Surprisingly, these animals showed normal locomotory function of body-wall muscle although the presence of certain subtle defects cannot be completely ruled out. On the other hand, it is still possible that the 15% of CSQ-1 level detected by western analysis (Fig. 10A) was contributed by body-wall muscles at levels that were undetectable by the GFP-tagged calsequestrin protein or immunostaining. Additionally, deficiency mutant embryos exhibit muscle twitching similar to wild-type, suggesting again that CSQ-1 is not essential for contraction (Lee at al., 1997a).

As observed in higher animals, it was our interest to determine whether calsequestrin interacts directly with the proteins involved in E-C coupling during muscle contraction in *C. elegans.* The results suggest that although UNC-68 interaction may be necessary for the proper localization of CSQ-1 in the sarcomeric membranes of *C. elegans* (Fig. 8A,B), the interaction may not be entirely required for body-wall muscle contraction (Table 1). In contrast to vertebrate skeletal muscles, there may be ryanodine receptor-independent contraction in *C. elegans* body-wall muscle as suggested by Maryon et al. (Maryon et al., 1998), where intracellular Ca^{2+} release is not essential for E-C coupling. One possible source of Ca^{2+} is intracellular stores from the ER and IP_3 receptor-dependent Ca^{2+} release. However, according to Santo et al. (Santo et al., 1999), IP_3 receptor in *C. elegans* is not expressed in body-wall muscle and mutants of the IP_3 receptor gene did not show any locomotory defects. Hence, this possibility seems unlikely. CSQ-1 is expressed in vulval muscle as well as in pharynx in *C. elegans*, which is consistent with UNC-68 expression. However, since these tissues were resistant to RNA interference, functional characterizations in these tissues were not possible. A function for CSQ-1 in these tissues should be revealed by the isolation of *csq-1* null mutants using a PCR-based screening of chemically mutagenized nematodes for deletion mutations (Liu et al., 1999).

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